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Identification of Genes involved in Iron Metabolism in Rhizobium leguminosarum ATCC 14479 Genome through the use of In-silico Analysis

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Identification of Genes involved in Iron Metabolism in *Rhizobium leguminosarum ATCC 14479* Genome through the use of *In-silico* Analysis

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Abstract

The complete genomic sequence of *Rhizobium leguminosarum ATCC 14479* has been determined. Its genome size is 7,935,223 base-pairs of DNA (bp). This multipartite genome contains 5 distinct replicons: a chromosome of 4,883,137 bp and four mega-plasmids of size 1,234,209 bp, 415,988 bp, 771,583 bp, and 630,306 bp. *In silico* (literally: *on computer*) analysis was done on the complete genome to detect genes relating to iron metabolism by bacteria. Seven iron-related operons and genes were found: nodulation genes, the Tol operon, the *hmuPSTUV* operon, iron response regulator genes, the *cycHJKL* operon, genes for bacterial cyclic glucans, and vicibactin genes.

Acknowledgements

I would like to start off by thanking my thesis mentor and professor Dr. Chakraborty for his endless motivation, continuous help, and invaluable knowledge he filled me with during the course of this research experience. I can't express enough how thankful I am for you for playing an incredible part of my journey to professional endeavor. Besides, I want to extend thanks to Dr. Lampson for always having the door open for me to ask questions, reading my thesis, and giving me valuable feedback. I would like to express my sincere appreciation to Sarah Zimmer and John Lusby for helping me understand the project and answering the many questions I have had. I want to thank the Health Science Honors-in-Discipline program and the College of Public Health for making this project feasible, offering me assistance in many forms, and giving me the chance to lead this exploration. Finally, I want to acknowledge my family, friends, fellow honors students, and all others who inspired me and helped me make this a worthwhile journey.

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Introduction

Rhizobia

Rhizobia are Gram negative, motile, rod-shaped, aerobic soil bacteria that belong to the alpha-Proteobacteria, a group consisting of species that form close associations with eukaryotic hosts [1]. They are known to live in a mutualistic symbiotic relationship with leguminous plants in which they induce formation of root nodules where nitrogen fixation takes place [2]. Legume seedlings germinate without any association to Rhizobia, but when nitrogen becomes limited in the environment, the plant and the bacteria start seeking each other out. The nitrogen-fixing bacteria supply fixed nitrogen (a nitrogen gas that has been converted to ammonia, an ammonium ion, nitrate, or another nitrogen oxide) for plants to use, while the plant provides nutrients and energy for the survival of bacteria [3].

Root nodule formation

One of the most intriguing bacterial-plant association is between Gram-negative bacteria and leguminous plants. These Gram-negative bacteria, which include *Rhizobium, Bradyrhizobium,* and other related genera, have the ability to induce root nodule formation in legumes [4].

Stages of rood nodule formation

1) The root cells of legumes release various chemicals such as flavonoids into the soil that stimulate growth of bacteria to high populations in the area around the roots called the *rhizosphere* [5].
2) Attachment factors help the bacteria recognize the correct host plant and to attach to their root hairs.
    a. A specific adhesion protein common on the surface of all Rhizobia is rhicadhesin. It binds to lectin receptors found on the surface of root hairs [6].
3) After binding to root hairs, the bacteria excrete substances known as Nod factors that stimulate the root hairs to curl [6].

4) The bacteria then penetrate the root hair through the tip and induce the plant to form a cellulosic tube, called the infection thread, through which the bacteria travel [6].
    a. This infection thread spreads down the root hair and into adjoining cells. Rhizobia multiply rapidly as they begin infecting the plant cells and form into cells with a different morphology called bacteroids. The rhizobia also release Nod factors that stimulate cell division/proliferation of root cells, leading to the formation of a new plant organ called a nodule [6].
5) The bacteroids are released from the infection thread into root cells via a process similar to exocytosis. During release, the bacteroids become encapsulated by the infection thread membrane which is continuous with the plant plasma membrane. This new membrane, which separates the bacteroids from the plant cell cytoplasm, is known as the symbiosome membrane. Within the symbiosome, the bacteroid is reliant on the legume for the supply of all nutrients that are needed for bacterial growth and nitrogen fixation. Besides, all nutrients must cross the symbiosome membrane before entering the bacteroid [6].

Genetics of rood nodule formation

Rhizobia contain *nod* genes that control different steps of the nodulation process. These genes are primarily found on symbiotic plasmids, in close proximity to *nif* genes which are responsible for nitrogen fixation [7]. Many nod genes have been identified in Rhizobial species, including *nodE, nodF, nodD, nodA, nodB, nodC, nodI*, and *nodJ* [8].

There are two modes of specificity for Rhizobium-legume symbiosis:

1. The first is the bacterial interaction with the flavonoids released by the plants. The flavonoids are sensed by the Rhizobial NodD protein. Different Rhizobial species produce different NodD proteins that can specifically interact with the flavonoids from their host [8].

The *nodD* gene encodes the NodD protein which is a transcriptional regulatory protein. NodD proteins become activated upon binding to their specific flavonoids. Upon activation, they bind to a region next to the *nodD* gene called the *nod-box* and increase transcription of other *nod* genes. These *nod* genes synthesize and modify nod factors, also called lipo-chitooligosaccharides [8].

2. *NodA, nodB*, and *nodC* genes are found in all Rhizobial species and function to synthesize a precursor compound of lipo-chitooligosaccharide [8]. The other *nod* genes are specific to certain Rhizobial species and they function to structurally modify the precursor compound in various ways. The final modified lipo-chitooligosaccharide is unique to a certain Rhizobial specie and responds specifically to its host plant. It leaves the bacterial cell via the NodI and NodJ proteins and induces the legume roots to form a nodule [8].

The interaction between nod factors and legumes was thought to be universal for a long time, but in 2007, it was proven that some photosynthetic *Bradyrhizobium* strains that are able to induce nitrogen-fixing nodules on *Aeschynomene* species lack the *nodABC* genes needed for nod factor synthesis. This showed that nodule organogenesis can be induced by an alternative mechanism, independent of nod factors [9].

Nitrogen fixation

Nitrogen is necessary for all living organisms, as it plays a crucial role in the synthesis of nucleic acids, proteins, ATP, and other nitrogen-containing compounds. Though nitrogen gas (N_2) makes up approximately 78% of the Earth's atmosphere, it is unusable by many organisms until it is fixed into usable forms such as ammonia and nitrates. The triple bond between the nitrogen atoms in N_2 makes it a stable molecule that is unable to take part in chemical reactions. Thus, breaking the bond and forming new bonds with hydrogen to make ammonia requires tremendous amount of energy. In fact, 16 molecules

of ATP and a powerful enzyme is required to make this happen [10]. The reduction reaction is shown in **Figure 1**:

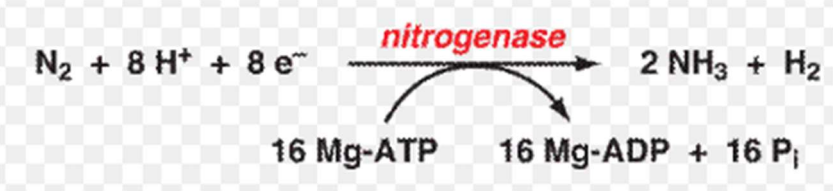


Figure 1: Nitrogen Fixation Reaction. Reduction of N_2 to NH_3

Nitrogen can be fixed in the following ways:

1. Biological Fixation: 60% of nitrogen is fixed through this way. Microorganisms convert atmospheric nitrogen into ammonia through the use of a nitrogenase enzyme [11].
2. Atmospheric Fixation: Only 5-8% is fixed through this way. Lightning spontaneously fixes nitrogen to form ammonia, which can be used to make fertilizers [12].
3. Industrial Fixation: This is accomplished by the human-induced Haber-Bosch process. Under high pressure (greater than 150 atm) and temperature of more than 500 degrees Celsius, N_2 is reduced to NH_3 [13].

The genes that control nitrogen fixation in rhizobia are known as *nif* genes. Free-living Rhizobia cannot fix nitrogen [12]. Only when they form a symbiotic relationship with the legumes and reside in the nodule will they fix nitrogen via the biological fixation mechanism. They use the nitrogenase enzyme to convert nitrogen gas into ammonia [12]. The legumes are benefitted since they can become independent of soil nitrogen. The Rhizobia (bacteroids) are benefitted since the legume provides them with a home to live in and necessary substrates (like carbohydrates from photosynthesis) required for survival. The bacteria utilize these nutrients to synthesize the high amount of ATP needed for nitrogen fixation [3].

Oxygen requirement and leghemoglobin

The bacteroids in the nodule require oxygen to make their ATP via cellular respiration [14]. However, the nitrogenase enzyme is sensitive to O_2 and will severely reduce its activity when coming into contact with it. The legume-Rhizobia symbiotes have an interesting mechanism to deal with this problem: synthesis of leghemoglobin. Leghemoglobin, which is only produced in fully formed nodules, is similar in structure and function to human hemoglobin. It functions to bind and regulate the levels of O_2 in the nodule. Leghemoglobin transports enough oxygen necessary for cellular respiration, but not too much that would lower the rate of nitrogenase enzyme [14]. Interestingly, both bacteria and legume contribute to the production of leghemoglobin: the legume produces the apoprotein and the bacterium produces the heme (an iron atom bound in a porphyrin ring) which forms the nonprotein part. The apoprotein and heme come together to make a functional leghemoglobin [15]. Leghemoglobin is found in the plant cytoplasm of infected cells [16].

Iron requirement

Iron is essential to nearly all forms of life, as it is involved in a wide variety of biological processes, including DNA biosynthesis, the tricarboxylic acid cycle, the electron transport chain, photosynthesis, nitrogen fixation, and oxygen transport [17]. Though it is the fourth most abundant element on the Earth's surface (oxygen, silicon, and aluminum are first, second, and third, respectively), under aerobic conditions and physiological pH, the ferrous form (Fe^{2+}) is readily oxidized to ferric (Fe^{3+}) form which forms insoluble complexes that become inaccessible to organisms such as soil bacteria [18]. Hence, microorganisms must employ various techniques to secure sufficient supplies of iron from their environment.

Since life began on Earth, the atmosphere has stayed aerobic, due to the rise of photosynthesis. The formula for photosynthesis is shown in **Figure 2** below:

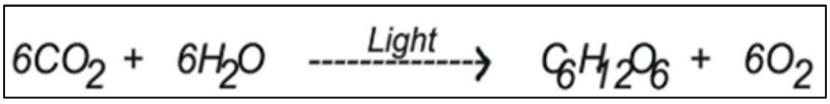


Figure 2: Photosynthesis formula

The aerobic environment has made iron much less soluble for the microorganisms to satisfy their nutritional needs. One way they bypass this problem is through the production and release of high-affinity, low molecular weight iron chelators known as siderophores [19]. These siderophores scavenge iron from the environment and form water-soluble complexes with Fe^{3+} that are able to be taken up by microorganisms. The association constant of siderophores for ferric iron is between 10^{12} and 10^{52} [19].

The presence of an outer membrane places an additional burden on Gram negative bacteria when bringing iron into the cell. The outer membrane is impermeable to hydrophilic molecules like siderophore-iron complexes that are too large (greater than 600 Daltons) to pass through the porins [20]. Therefore, the bacteria have established different import systems, consisting of outer membrane receptors and cytoplasmic membrane proteins TonB and ExbBD that work in an energy-dependent manner [20]. No energy source is present at the outer membrane; the energy for transport through the outer membrane is provided by the inner membrane complex made up of TonB, ExbB, and ExbD proteins [20]. This is known as the TonB-dependent transport. When the ligand (which could be Vitamin B12, heme, iron-siderophore and nickel-nickelophore complexes, and some carbohydrates) binds to its outer membrane receptor, TonB/ExbB/ExbD complex uses the proton motive force of the cytoplasmic membrane to change the conformation of the receptor that lets the ligand into the periplasm [20]. The conformational change is the result of a physical interaction between C-terminal domain of TonB protein and the receptor [20]. Once the ligand is in the periplasm, a periplasmic-binding protein binds to it and delivers it to the inner membrane permease complex, which transports the ligand into the cytoplasm in an ATP-dependent manner [20].

A single TonB can transduce energy to more than one receptor. It has been proposed that a consensus sequence called the TonB box exists in the outer membrane receptors and it is this TonB box that interacts with the TonB protein [20]. ExbB and ExbD are both necessary for TonB dependent uptake [20].

Mutation in either of these proteins can, however, be complemented by TolQ or TolR, proteins of the Tol system that show homology to ExbB and ExbD proteins, respectively [21].

Tol system

The Tol system consists of 7 genes (*ybgC, tolQ, tolR, tolA, tolB, pal*, and *ybgF*) that are organized in a gene cluster, which is transcribed as two different operons [22]. The first transcript consists of *ybgC, tolQ, tolR,* and *tolA*, while the second transcript consists of *tolB, pal*, and *ybgF* [22]. TolQ, TolR, and TolA are found in the inner membrane; TolB and YbgF are found in the periplasm; Pal is an outer membrane-linked lipoprotein; YbgC is found in the cytoplasm. Like TonB, TolA is responsible for providing energy via the proton motive force present across the inner membrane [22]. The energy is most likely used to maintain outer membrane stability. Mutations to *tol* genes have caused bacteria to develop hypersensitivities to antibiotics and detergents that compromise outer membrane integrity and stability [23]. The periplasmic proteins are also found to be leaked out of the mutant bacteria [24]. Besides, in mutant bacteria, there is production of outer membrane vesicles [25] and a defect in O-antigen polymerization [26]. These phenotypes support the fact that the Tol system has a role, in part, in maintaining outer membrane stability. More research is currently being done.

Iron deficiency and its effect on Rhizobium-legume symbiosis

Legumes that are able to form a symbiosis with nitrogen-fixing bacteria have a higher demand for iron [27]. There is a positive correlation between the rate of nitrogen fixation in common bean plant nodules and the iron concentration in nodules [28]. Multiple studies have been done to discover the relationship between iron deficiency and nodule formation. Peanuts, chickpeas, lupins, lentils, soybean, and French beans have been explicitly studied [27]. When *Lupinus angustifolius* (blue lupine) is grown under conditions of iron deficiency, fewer nodules form, indicating an effect on nodule initiation [27]. But in peanuts, common bean, and soybean, iron deficiency has no effect on the nodule initiation but does affect later nodule development [29]. It was figured that peanuts that were grown in iron-deficient conditions

had up to 215 times fewer bacteroids within the infected region, giving support to the idea that iron plays a role in the differentiation of Rhizobia and the development of the resulting bacteroides [29].

The need for iron in the Rhizobium-legume symbiosis

Iron is a crucial component of many proteins required in the symbiosis. This large requirement for iron is evident in the fact that at nodule maturity, soybean nodules contain nearly 44% of total iron in the plant, compared to 31% in leaves, 7% in seeds, and 5% in roots [30].

1. Ferredoxin: an iron-sulfur protein that, through oxidation, transfers electrons to the nitrogenase enzyme [31].
2. Nitrogenase: an enzyme responsible for fixing nitrogen. It is composed of two components, both of which require iron [31].
    a. Ferredoxin transfers electrons to the Fe-protein (smaller component), which transfers them to the molybdenum-iron protein (larger component). This larger component contains the catalytic site, where N_2 binds, receives the transferred electrons, and gets reduced [32].
3. Cytochrome components: Iron is required in these components of the bacterial electron transport chain which provides energy for nitrogen fixation [33].
4. Leghemoglobin: Bacterial respiration requires oxygen, but the nitrogenase enzyme is sensitive to oxygen. This conflicting demand is solved through the production of leghemoglobin, proteins similar to hemoglobin, that bind and regulate O_2 levels in the nodule [14]. 24% of the total iron in the nodule is present in leghemoglobin [34].

Iron transport to the nodule

Iron is supplied to the nodule primarily by xylem whose low pH supports ferric citrate complexes [35]. But it has also been found that the surface of the nodule contains ferric citrate reductase that allows iron uptake directly from soil [36].

Uptake of iron: free-living Rhizobia vs. nitrogen-fixing bacteroids

Free-living Rhizobia take up iron from the environment via different ways. These include the release of siderophores, the release of hemophores to utilize iron from heme, and the reduction of Fe^{3+} to Fe^{2+} and taking in that Fe^{2+} [37]. Nitrogen-fixing bacteroids can take up Fe^{2+} and Fe^{3+}via transporters not yet characterized [38]. Ferric chelators, such as siderophores, are not the major source of iron transport into the bacteroids, as shown by downregulation experiments [39, 40]. However, the chelators can serve to bind and store Fe^{3+} in the symbiosome space. The ferric iron, when needed, can be reduced to Fe^{2+} before being taken up by the bacteroid [38]. Heme might also not be a major source of iron for bacteroids, as they don't have direct contact with leghemoglobin found in the plant cytoplasm of infected cells [41]. However, during nodule senescence, the nodule cytoplasm becomes acidic and this causes the release of heme from leghemoglobin [42]. Besides, the symbiosome membrane degrades at this time, so the bacteroids can have direct contact with heme. Heme uptake is similar to siderophore uptake and even a high affinity heme-binding outer membrane protein has been found on the surface [43].

The hmuPSTUV operon

This operon, made of five genes, is responsible for the utilization of heme as a source of iron. In Rhizobia, the expression of this operon is enhanced in iron-limiting conditions and *hmu* mutants showed only a slight decrease in growth. This slight decrease means Rhizobia are able to use a different system than *hmuPSTUV* to acquire heme [40].

Iron response regulators

RirA

The *rirA* gene codes for a Rhizobial iron regulator RirA that acts as a repressor under iron-replete conditions. The *rirA* gene is downregulated under iron-deplete conditions by IrrA [44]. Mutations in the *rirA* gene caused high-level continuous expression of several operons which are normally responsive to iron concentration and whose products are involved in the uptake of iron, including

heme [44]. However, *rirA* mutants showed reduced growth in iron-replete conditions, not because of iron-deficiency but because of toxic levels to which iron was transported into bacteria [45]. The genes under negative regulation by RirA include *hmuPSTUV, tonB, fhu, VbsC,* and *rpol* [46].

The RirA protein works as a repressor under iron-replete conditions by binding to Iron-Responsive Operator sequences (5'-TGA-(N9)-TCA-3'), also known as IRO boxes or RirA boxes [47]. It has been found that the RirA protein and a [4Fe–4S] cluster combine to form a holoprotein, and this holoprotein is what binds to IRO boxes. In conditions of iron deficiency, the [4Fe–4S] cluster converts into [2Fe–2S] cluster, and this conversion causes the RirA protein to not bind to IRO boxes, thus increasing transcription of the RirA-controlled genes [48].

Irr

The Irr (Iron Responsive Regulator) protein belongs to the Fur superfamily and only works in iron-deficient conditions. In iron-deficient conditions, Irr binds to Iron-Control Element (ICE) motifs and represses genes that are active in iron-replete conditions [49]. When iron becomes available, Irr becomes inactive, in a heme-dependent manner [50]. Heme directly binds to Irr and inhibits it [50]. The *rirA* gene is, in fact, controlled by Irr. The Irr protein binds and inhibits transcription of *rirA* gene under iron-deficient conditions [47]. ICE consensus sequence is determined to be TTTAGAA-N_3-TTCTAAA [46].

The cycHJKL operon and the feuPQ genes

The *cycHJKL* operon of *Rhizobium leguminosarum* is known to be responsible for the maturation of cytochrome c, possibly by its involvement in the covalent attachment of heme to the apoprotein. *CycHJKL* mutants are thought to have lost a high affinity iron acquisition system. They don't undergo nitrogen fixation, fail to create and export siderophores, cause accumulation of protoporphyrin IX (the immediate precursor of heme) in the cell, and cause reduction in levels of holocytochrome in the periplasm [51]. Upstream of the operon is a *lipA* gene which encodes an outer membrane lipoprotein [52].

Further upstream of *lipA* gene are two genes that are known to be responsible for two-component regulation. *FeuP* codes for a response regulator and *feuQ* codes for a sensor protein [53]. Mutation in these two genes cause inability to take up Fe^{3+} from the environment [53]. However, FeuP and FeuQ neither affected the transcription of *lipA* nor that of the *cycHJKL* operon [53]. A mutation in *feuQ* led to a loss of the high affinity iron uptake system, although siderophores were still produced [53].

The FeuQ protein is a sensor protein in the cytoplasmic membrane that monitors the cell environment and sends signals to FeuP [53]. In low osmolarity conditions of the periplasm, FeuQ will phosphorylate FeuP, which will bind to DNA and increase transcription of the genes it controls [53]. FeuP is known to control at least 16 genes [54]. One of those genes is *ndvA* (see below) [54].

Bacterial cyclic glucans and the ndvA and B genes

Cyclic beta-(1,2)-glucans are periplasmic components that serve as storage sites for excess iron [55]. They sequester ferrous iron to protect bacteria from iron-induced toxicity under iron-replete conditions and to provide iron to bacteria under iron-deplete conditions [55]. The ferrous iron is easily able to diffuse through the porins in the outer membrane into the periplasm where the excess is sequestered [55]. When needed, iron can be transported from the periplasm into the cytoplasm via Ferrous iron transporters (Feo) located in the cytoplasmic membrane [55].

One of the genes activated by FeuP is *ndvA*, which codes for a glucan transporter across the cytoplasmic membrane [54]. *FeuP* mutants have been discovered to have decreased cyclic beta glucans in the periplasm and thus decreased growth under iron-limiting conditions [54]. The low osmolarity detected by FeuQ is what activates FeuP to stimulate *ndvA* expression. *NdvB* gene is responsible for the synthesis of glucans [54].

Present Work

Rhizobium leguminosarum ATCC 14479 was the strain used for this research. Previous researchers in our lab have found various iron transport systems in rhizobia, such as the use of siderophores like vicibactin

to acquire iron from the environment. The utilization of heme as the source of iron was also proven, by Sushant Khanal who, along with Sarah Zimmer, sequenced the five genes of the *hmuPSTUV* operon. This operon is responsible for producing products involved in the uptake of heme from the environment.

Recently, our lab was successful in sequencing the entire genome of this strain (*ATCC 14479*). Thus, it was of our interest to go through the entire genome to confirm the genes for the work that has already been done like Vicibactin genes and the *hmuPSTUV* genes and to identify other iron-related genes.

Methods

In-silico analysis was used for this research, meaning everything was done on a computer. The *NCBI sequence database* was used to obtain the genomic DNA sequence of *Rhizobium leguminosarum ATCC 14479*. This DNA sequence was subjected to *sequence analysis* which is the process of studying a DNA, RNA, or protein sequence using analytical methods to understand its function. *Sequence alignment* is one such method; it was used to compare the DNA segment in question to another DNA segment to identify regions of similarity; similarity was used to assign a function to the DNA in question. Searches against biological databases was done using the *NCBI BLAST*, which finds regions of similarity between biological sequences by comparing DNA (or protein) sequences in question to other sequence databases.

The advancement in technology has led to an exponential increase in the number of new sequences added to the sequence database. However, by themselves, the sequences don't give much information. They need to be further analyzed using genomic comparisons and sequence alignments with sequences of known function. The high similarity between a gene sequence in our bacteria and a gene sequence with known function from another bacteria was used to assign that same function to the gene in question in *Rhizobium leguminosarum ATCC 14479*. The *NCBI Blast* provides this data through the similarity match with amino acids of organisms already present in the database.

Results and Discussion

Nod genes (Plasmid 2)

Nodulation is important to consider because nitrogen fixation by rhizobia takes place in the nodule. The enzyme responsible for nitrogen fixation is nitrogenase and it requires iron to function. The process of nodulation is also iron-dependent. The development of a nodule requires an interaction between both species. Plants release flavonoids that stimulate the rhizobia to produce NodD (inactive). NodD becomes activated upon binding to flavonoids. The complex binds to a site known as nodD box. This stimulates the transcription of other *nod* genes. The nod genes are responsible for creating a nod factor (also called lipo-chitooligosaccharide) that will initiate root curling and formation of an infection thread.

The bacteria move along the infection thread and invade root cells. Root cells differentiate into a nodule, and rhizobia differentiate into bacteroids which can fix nitrogen. In *R. leguminosarum ATCC 14479,* nodulation genes were found on Plasmid 2, in between genes responsible for nitrogen fixation (*nif/fix*) as shown in **Figure 3** below:

CDS number	**Location**	**Gene/ function**
WP_063474979.1	50..1,582	*NifK*, nitrogenase molybdenum-iron protein subunit beta
WP_063474980.1	1,635..3,074	*NifE*, nitrogenase iron-molybdenum cofactor biosynthesis protein
WP_063474981.1	3,080..4,417	*NifN*, nitrogenase iron-molybdenum cofactor biosynthesis protein
WP_063474914.1	30,946..32,109	iron-containing alcohol dehydrogenase
WP_063474919.1	42,039..42,953	4Fe-4S binding protein
nitrogen fixation protein FixA	46,181..46,270	*FixA*, nitrogen fixation protein, pseudo partial stop
WP_063474928.1	72,395..72,628	Nif11-like leader peptide family natural product precursor
WP_112907837.1	85,484..86,662	iron-containing alcohol dehydrogenase

WP_003549664.1	100,160..100,312	*FixQ/CcoQ* family Cbb3-type cytochrome c oxidase assembly chaperone
iron-containing alcohol dehydrogenase	333,982..334,308	iron-containing alcohol dehydrogenase, pseudo partial stop
nitrogen fixation protein FixA	336,777..336,866	*FixA*, nitrogen fixation protein, pseudo partial stop
WP_082849715.1	387,420..387,713	ferredoxin III, nif-specific
WP_063474964.1	388,804..389,538	cysteine hydrolase
WP_063474965.1	389,560..390,927	sulfonate ABC transporter substrate-binding protein
WP_063474966.1	391,419..392,522	acyltransferase
WP_017968974.1	392,809..393,300	*NodN*, MaoC family dehydratase
WP_063474967.1	393,380..395,206	*NodM*, GlmS, glutamine--fructose-6-phosphate transaminase (isomerizing)
WP_017968972.1	395,642..396,193	*NodL*, sugar O-acetyltransferase
hypothetical protein	396,337..396,576	Pseudo, Partial stop Comment: incomplete; partial in the middle of a contig; missing stop
WP_063474968.1	396,679..397,887	*NodE*, beta-ketoacyl-[acyl-carrier-protein] synthase family protein
WP_033181209.1	397,887..398,165	*NodF* nodulation protein
WP_063474969.1	398,708..399,655	*NodD*, transcriptional regulator
WP_081295649.1	399,882..400,472	*NodA* family N-acyltransferase

WP_082849712.1	400,469..401,119	*NodB*, chitooligosaccharide deacetylase
WP_063474972.1	401,140..402,420	*NodC*, chitooligosaccharide synthase
WP_082849713.1	402,459..403,487	*NodI*, nodulation factor ABC transporter ATP-binding protein
WP_063474973.1	403,484..404,272	*NodJ* nodulation protein
IS3 family transposase	404,489..405,587	IS3 family transposase
WP_032491863.1	405,691..405,903	*NifT/FixU* putative nitrogen fixation protein
WP_082849714.1	406,083..406,277	*FdxN*, ferredoxin
WP_063474974.1	406,295..407,758	*NifB*, nitrogenase cofactor biosynthesis protein
WP_063474975.1	407,993..409,078	*NifA*, nif-specific transcriptional activator
WP_063474976.1	409,279..409,575	*FixX*, Ferredoxin family protein
WP_063474977.1	409,588..410,895	*FixC*, FAD-binding protein
WP_032491865.1	410,906..412,018	*FixB*, electron transfer flavoprotein subunit alpha
WP_032491857.1	412,035..412,883	*FixA*, electron transfer flavoprotein subunit beta
WP_003563197.1	413,446..414,339	*NifH*, nitrogenase iron protein
WP_063474978.1	414,440..415,960	*NifD*, nitrogenase molybdenum-iron protein alpha chain

Figure 3: Locations of *nod* genes and the surrounding genes in *R. leguminosarum ATCC 14479*. *Nod* genes are highlighted in blue. *Nif/fix* genes, responsible for nitrogen fixation, surround the nod genes.

NodA, B, and *C* genes are found in all rhizobia and they function to synthesize a precursor nod factor (lipo-chitooligosaccharide). *Nod E* and *F* function to modify that precursor into a specific product that can interact specifically with its host legumes. *Nod I* and *J* are responsible for the export of the nod factors from the bacteria. *Nod L, M,* and *N* are known to suppress nodulation.

The nod gene cluster in our species is shown in **Figure 4** below:

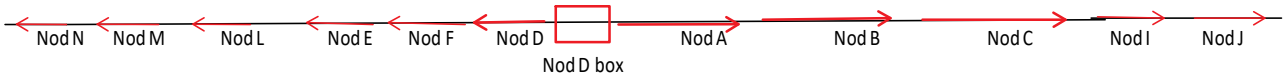


Figure 4: Nod gene cluster in *R. leguminosarum ATCC 14479*

The Tol system (Chromosome)

Ferric-siderophore complexes are too large to pass through the outer membrane porins, thus they must be taken up by a different mechanism. Rhizobia, like other Gram-negative bacteria, contain an outer membrane protein. There is no energy source in the outer membrane. The TonB-ExbB-ExbD complex uses the proton motive force present across the cell membrane to provide energy to the outer membrane. As a result, the outer membrane protein changes conformation and lets the complex in. It then goes through the ABC transporter complex into the cytoplasm.

Heme, vitamin B12, and nickel-nickelophore complexes all undergo a similar transport mechanism. When the ligand binds to the outer membrane protein, TonB, ExbB, and ExbD work together to provide energy for its transport. However, research has shown that mutations in either ExbB and ExbD can be complemented by TolQ and TolR, respectively. The latter are the proteins of the Tol system.

The Tol system consists of seven genes 7 genes, synthesized as two different transcripts as shown in **Figure 5** below:

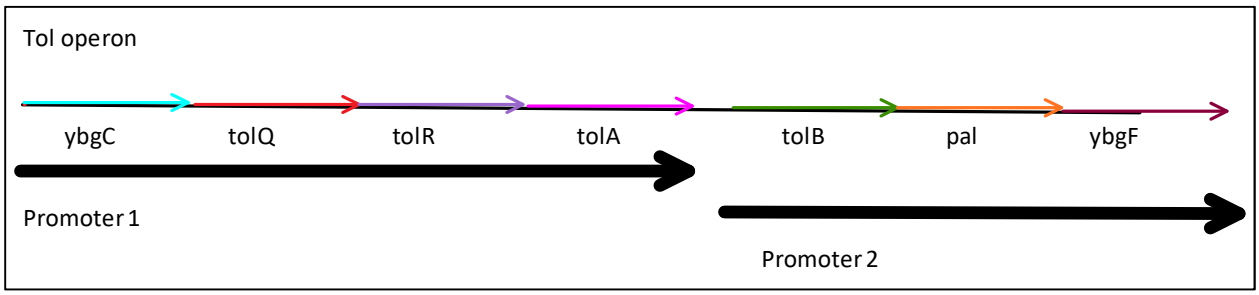


Figure 5: *Tol* gene cluster in *R. leguminosarum ATCC 14479*

The operon is responsible for maintaining outer membrane stability. Research has shown that mutations in *tol* genes have resulted in hypersensitivities to antibiotics and detergents. The periplasmic proteins are also found to be leaked out of the mutant bacteria.

The specific locations of the *tol* genes in *R. leguminosarum ATCC 14479* are shown in **Figure 6** below:

CDS number	Location	Gene/ function
WP_112904918.1	2,188,270..2,189,259	YbgF, tol-pal system protein
WP_018243435.1	2,189,423..2,189,956	Pal, peptidoglycan-associated lipoprotein
WP_026188517.1	2,190,110..2,191,420	TolB, tol-pal system protein
WP_112904920.1	2,191,442..2,192,578	hypothetical protein
WP_017968102.1	2,192,587..2,193,039	TolR protein
WP_003542733.1	2,193,058..2,193,777	TolQ protein
WP_018243438.1	2,194,153..2,194,608	ybgC, tol-pal system-associated acyl-CoA thioesterase

Figure 6: Location of *tol* genes in *R. leguminosarum ATCC 14479*

The following **Figure 7** is the NCBI data which shows that all of these genes are in a linear fashion:

Figure 7: NCBI data of *tol* genes in *R. leguminosarum ATCC 14479*

The hmuPSTUV operon (Chromosome)

Previous research in our lab has shown that our bacteria can utilize heme as a source of iron, as heme contains iron. The operon responsible for this is *hmuPSTUV*, which is TonB-dependent and consists of the five genes: *P, S, T, U*, and *V*.

The expression of this operon is enhanced in iron-limiting conditions. In iron-replete conditions, it is actually repressed by RirA.

The following **Figure 8** shows the locations of the *hmuPSTUV* genes, along with those genes that surround them:

CDS number	Location	Gene/ function
WP_112904717.1	1,962,718..1,963,032	antibiotic biosynthesis monooxygenase
WP_112904719.1	1,963,042..1,963,314	Hypothetical protein
WP_018243248.1	1,963,311..1,963,718	DUF2325 domain-containing protein
WP_112906647.1	1,963,826..1,965,157	TonB protein
WP_112906648.1	1,965,213..1,965,635	Hypothetical protein
WP_012758649.1	1,965,810..1,965,992	heme uptake protein HmuP
WP_112904721.1	1,966,001..1,967,050	heme degrading factor HmuS
WP_112906649.1	1,967,062..1,967,991	Periplasmic heme binding protein HmuT
WP_112904723.1	1,967,994..1,969,106	heme ABC transporter permease HmuU
WP_112904725.1	1,969,143..1,969,937	ATPase component of heme ABC transporter HmuV
WP_018243255.1	1,970,085..1,970,429	DUF883 family protein
WP_112904727.1	1,970,439..1,970,840	Hypothetical protein
WP_003542247.1	1,971,092..1,971,649	RNA polymerase sigma factor
WP_024319398.1	1,971,660..1,971,866	Hypothetical protein
WP_018243259.1	1,972,123..1,972,917	Response regulator
WP_112906650.1	1,973,120..1,974,106	Sensor histidine kinase

Figure 8: Locations of the *hmuPSTUV* and surrounding genes in *R. leguminosarum ATCC 14479*

The following **Figure 9** shows the NCBI data which shows that TonB is transcribed divergently from the *hmuPSTUV* operon. Previous research had shown that TonB and the hypothetical protein are co-transcribed divergently from the *hmuPSTUV* operon:

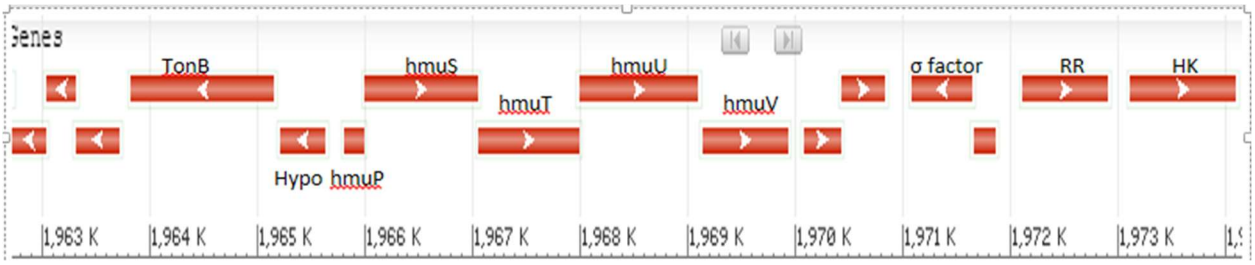


Figure 9: NCBI data of the *hmuPSTUV* genes in *R. leguminosarum ATCC 14479*

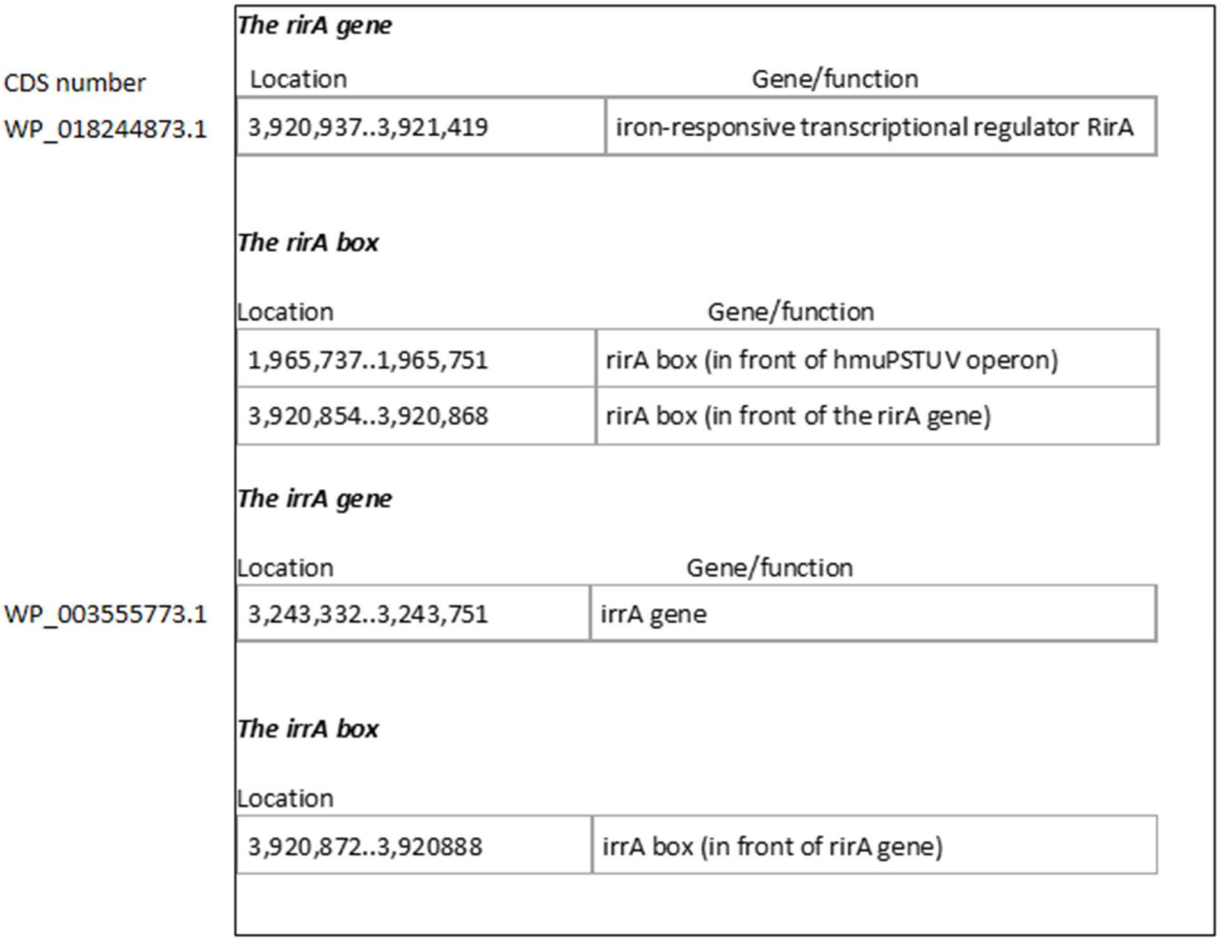
The iron response regulators (Chromosome)

There are two main iron response regulators in *Rhizobia*. RirA acts as a repressor under iron-replete conditions and IrrA acts as a repressor under iron-deplete conditions.

RirA is known to control genes like *hmuPSTUV, tonB, vicibactin, irr, rirA,* etc. by binding to IRO sequences (rirA box) upstream of the genes. IrrA is known to control genes like *irr, rirA* etc. by binding to ICE motifs (irrA box) upstream of the genes.

The following **Figure 10** shows the location of *rirA* gene, rirA boxes, the *irrA* gene, and the irrA box in *R. leguminosarum ATCC 14479*. There are obviously more of the boxes in the genome, as there is one

upstream of every gene the repressors control:



The rirA gene

CDS number	Location	Gene/function
WP_018244873.1	3,920,937..3,921,419	iron-responsive transcriptional regulator RirA

The rirA box

Location	Gene/function
1,965,737..1,965,751	rirA box (in front of hmuPSTUV operon)
3,920,854..3,920,868	rirA box (in front of the rirA gene)

The irrA gene

Location	Gene/function
3,243,332..3,243,751	irrA gene

The irrA box

Location	
3,920,872..3,920888	irrA box (in front of rirA gene)

Figure 10: Locations of *rirA* gene, rirA boxes, *irrA* gene, and irrA box in *R. leguminosarum ATCC 14479*

The following **Figure 11** shows the *rirA* gene in purple:

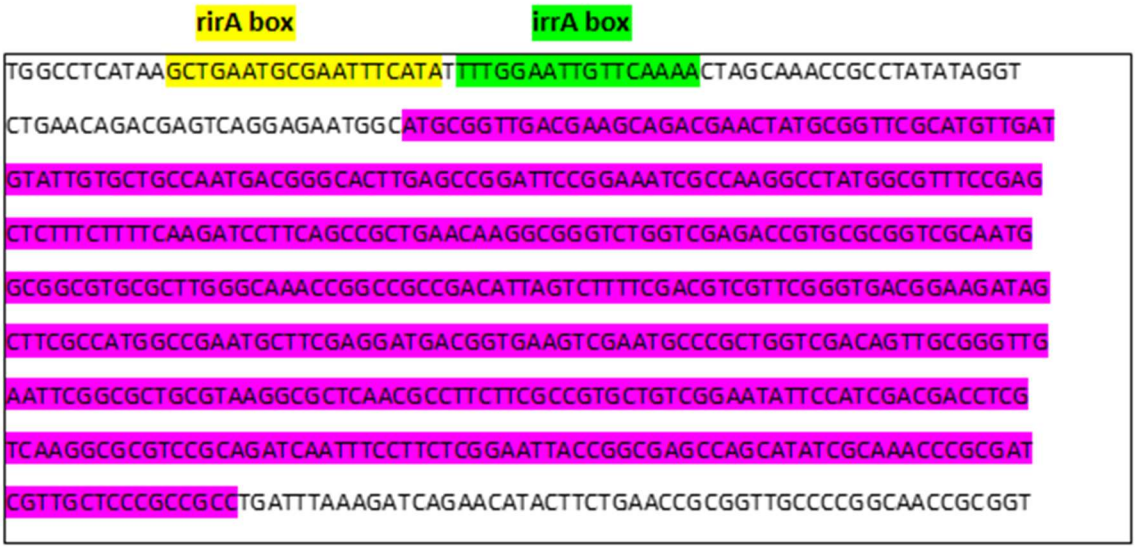


Figure 11: The *rirA* gene sequence and its two cis-acting elements in *R. leguminosarum ATCC 14479*

As can be seen in the figure above, *rirA* gene contains two cis-acting elements upstream of the gene: a rirA box in yellow and an irrA box in green. This means that the gene is negatively autoregulated by RirA under iron-replete conditions and repressed by IrrA under iron-deplete conditions.

The cycHJKL operon and the feuPQ genes (Chromosome)

Research concerning the *cycHJKL* operon is still ongoing. But it has been shown that *cycHJKL* mutants have shown to have phenotypic defects such as inability to fix nitrogen, inability to synthesize and export siderophores, accumulation of precursors of heme in the cell, and reduction in levels of holocytochrome in the periplasm. This supports the idea that *cycHJKL* mutants probably have lost a high affinity iron acquisition system.

Upstream of the operon is a *lipA* gene which codes for an outer membrane protein. Upstream of *lipA* are two genes: *feuP* and *feuQ*. FeuQ is a sensor histidine kinase protein in the cell membrane and FeuP is a response regulator. In low osmolarity conditions of the periplasm, FeuQ will phosphorylate FeuP, which will bind to DNA and increase transcription of the genes it controls. FeuP is known to control at least 16

genes, of which one is the *ndvA* gene responsible for the export of glucans to the periplasm **(more information detailed below)**.

The following **Figure 12** shows the location of the *cycHJKL* and *feuPQ* genes:

CDS number	Location	Gene/function
WP_018241306.1	4,590,661..4,591,332	FeuP, response regulator transcription factor
WP_003546769.1	4,591,322..4,592,725	FeuQ, HAMP domain-containing histidine kinase
WP_018446204.1	4,592,844..4,593,266	lipA protein
WP_112906391.1	4,593,418..4,594,605	cycH, c-type cytochrome biogenesis protein CcmJ
WP_112906392.1	4,594,602..4,595,099	cycJ, cytochrome c maturation protein CcmE
WP_112906393.1	4,595,096..4,597,093	cycK, heme lyase CcmF/NrfE family subunit
WP_112906394.1	4,597,090..4,597,545	cycL, cytochrome c-type biogenesis protein CcmH

Figure 12: Locations of the *cycHJKL* and *feuPQ* genes in *R. leguminosarum ATCC 14479*

The genes are all in a linear fashion, as can be seen in **Figure 13** below:

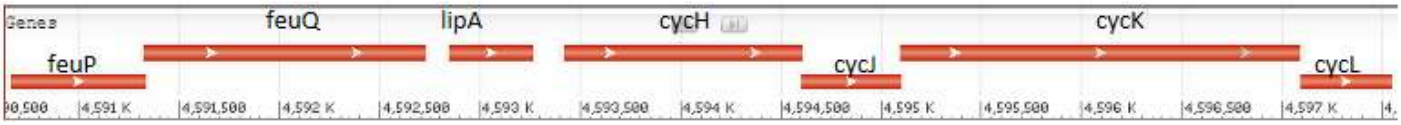


Figure 13: NCBI data of the *cycHJKL* operon and the upstream *lipA* and *feuPQ* genes

Bacterial cyclic glucans and the ndv genes (Chromosome)

Cyclic beta-(1,2)-glucans of the periplasm function to store excess iron. Under iron-replete conditions, they sequester iron since too much iron is toxic, and under iron-deplete conditions, they allow that iron to be used by the bacteria. The *ndvB* gene is responsible for the synthesis of glucans, and the *ndvA* gene is responsible for the export of those glucans to periplasm.

The following **Figure 14** shows the specific locations of these genes:

CDS number	Location	Gene/ function
WP_112906692.1	3,005,700..3,007,463	ndvA protein
WP_112905540.1	3,009,879..3,018,398	ndvB protein

Figure 14: Locations of the *ndvA* and *ndvB* genes in *R. leguminosarum ATCC 14479*

Vicibactin genes (Plasmid 1)

Vicibactin is an important siderophore used by rhizobia and has been extensively studied in our lab. The eight genes responsible for its synthesis were found on Plasmid 1. There was also a *fhuF* iron-reductase gene and *TonB* dependent siderophore receptor gene present near the gene cluster.

This is shown in **Figure 15** below:

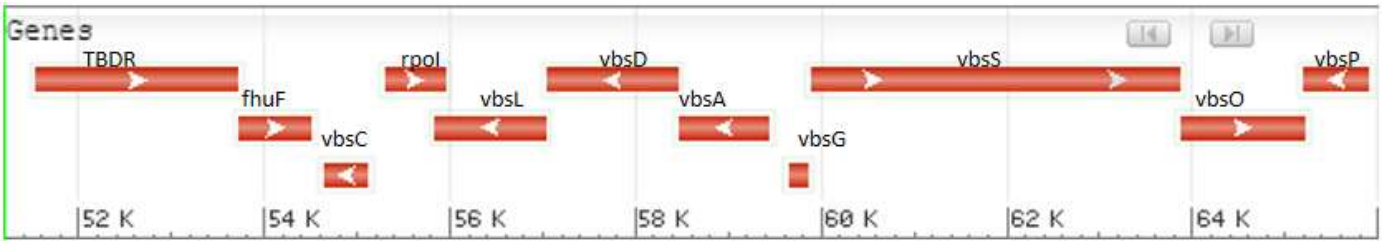


Figure 15: NCBI data of Vicibactin genes in *R. leguminosarum ATCC 14479*

The following **Figure 16** shows the location of the Vicibactin genes:

CDS number	Location	Gene/function
WP_112906815.1	51,541..53,721	*fhuA*, TonB-dependent siderophore receptor
WP_112906816.1	53,725..54,513	*fhuF*, siderophore-iron reductase
WP_112906817.1	54,635..55,126	*vbsC*, GNAT family N-acetyltransferase
WP_112906818.1	55,291..55,950	*rpoI*, sigma-70 family RNA polymerase sigma factor
WP_112906819.1	55,841..57,028	*vbsL*, hypothetical protein
WP_112906820.1	57,039..58,460	*vbsD*, MATE family efflux transporter
WP_112906821.1	58,457..59,446	*vbsA*, acetyltransferase
WP_112906822.1	59,640..59,858	*vbsG*, MbtH family NRPS accessory protein
WP_112906823.1	59,878..63,870	*vbsS*, amino acid adenylation domain-containing protein
WP_112906824.1	63,867..65,219	*vbsO*, lysine 6-monooxygenase
WP_112906825.1	65,192..65,908	*vbsP*, 4'-phosphopantetheinyl transferase superfamily protein

Figure 16: Locations of the Vicibactin genes in *R. leguminosarum ATCC 14479*

Conclusion

Iron is one of the most important metals as it acts as a cofactor for many enzymes and proteins. What we have learned is that, despite being an abundant element, iron is in short supply to microorganisms because of the insolubility of the complexes it forms under physiological conditions. To circumvent this limitation, microorganisms have come up with ways to acquire iron from the environment and even store

it for later use. One powerful method is the secretion of high-affinity iron chelating molecules called siderophores that bind to and transport iron into the organism.

The research on rhizobia can be applicable broadly to many other organisms, including mammals. In humans, for instance, how is the iron that is found in blood replaced when blood is lost through a cut? This prompts us to consider ways that iron can be transported from the environment or utilize iron from storage sites.

Myoglobin and Hemoglobin are required for the transport of oxygen in humans. Their tertiary structure is especially important. When the structure of hemoglobin changes even slightly and thus no longer transports oxygen efficiently, a genetic disease known as thalassemia results. Patients with thalassemia have similar symptoms to anemic patients, as they both technically have low healthy blood cell count.

The most common treatment for thalassemia is blood transfusion. However, this still comes with problems. Although the healthy red blood cell count is normalized through blood transfusion, with every blood transfusion comes additional iron which builds up more and more, causing iron overload or hemochromatosis. This overload is toxic to humans and can promote bacterial infection. The bacteria can take in that additional iron through siderophores, for example, and use it to their advantage to grow and infect. As was discussed in this paper, too much iron is toxic for rhizobia and thus iron-related genes are highly regulated and they even contain cyclic glucans in their periplasm to sequester excess iron. Too less iron is not good either! Iron is required for so many growth processes. The key is homeostasis.

Rhizobial *ndvB* gene codes for the synthesis of cyclic glucans and *ndvA* gene codes for the export of glucans into periplasm where they serve as storage sites to maintain homeostasis. Humans must also have a means to store iron; what would happen if iron is suddenly lost from heavy bleeding? To prevent damage from excessive loss, iron is stored in the bone marrow, spleen, and liver which can provide access to iron under limited conditions. In fact, about 10 percent of all the iron in the body is in storage, with ferritin and hemosiderin serving as iron-storage proteins.

My research was a theoretical research to identify iron-related genes. Now actual experiments can be done on all of those genes. Knock-out genes can be performed for each of the findings of this research to see their effect on rhizobia. The research on *cycHJKL* genes is still ongoing and though the products of the genes are believed to be important in cytochrome maturation, it will be interesting to find the exact function of each of the genes. This paper and its findings basically open up more doors for future researchers, as now, the whole sequenced genome has been analyzed for major iron-related operons and genes.

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